

## THE QUANTITATIVE DEPENDENCE OF GLUCOSE-6-PHOSPHOHYDROLASE UPON PHOSPHOLIPIDS: EFFECTS OF PHOSPHOLIPASE C AT 5° AND 37°

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### 1. Introduction

Since the classical, pioneering studies of Beaufay and De Duve [1], it has been known that glucose-6-phosphohydrolase (G-6-Pase) is a phospholipid-dependent enzyme. However, the specificity of this dependence is still unknown. Duttera et al. [2] found that *Cl. welchii* phospholipase C, which hydrolyses microsomal phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin, causes 80–90% inhibition of G-6-Pase. Addition of PE or lysolecithin regenerated G-6-Pase activity in phospholipase treated microsomes but PC did not.

Our investigations of the quantitative dependence of G-6-Pase upon PC and PE show that in microsomes treated with phospholipase C at 37° there is a fairly close correlation between PC hydrolysis (90–100%) and loss of G-6-Pase activity (95–100%), but no apparent relationship with PE hydrolysis which plateaus at 50–70%. When, however, phospholipase C treatment was done at 5° the situation was reversed: G-6-Pase plateaued at 40–60% of its initial activity like PE, while 90–95% PC hydrolysis was still obtained as at 37°. In addition, unlike the situation after phospholipase treatment at 20° [2], PC completely restored the G-6-Pase activity lost after 5° treatment.

The different effects of phospholipase C treatment at 5°, 20° and 37° do not reflect difference in phospholipid hydrolysis, but are related to heat inactivation. Postincubation at 20° or 37°, of 5° phospholipase treated microsomes after the addition of EGTA, leads to loss of G-6-Pase approximately proportional

to the extent of prior phospholipid depletion at 5°. Added PC prevents this heat inactivation but is unable to reverse it. It is concluded that phospholipid depletion methods will only yield unequivocal information on the phospholipid dependence of G-6-Pase if they are applied at low temperatures.

### 2. Experimental

Experiments were performed on 150–300 g male rats fasted 16–24 hr and injected with radioactive choline or ethanolamine 30 min before killing. Liver microsomes were prepared by centrifuging mitochondrial supernatants in 0.25 M sucrose at 105,000 g for 60 min. Phospholipase treatment was carried out at 5° and 37° with 500 µg/ml of phospholipase C (*Cl. welchii*, Sigma) dissolved in 1% bovine serum albumin. Phospholipase incubations contained 1 mM CaCl<sub>2</sub> and 20 mM tris-maleate buffer, pH 6.8. Phospholipase action was terminated by making incubations 3.3 mM with respect to EGTA. This was shown to stop phospholipase action completely. Blank incubations were always run containing phospholipase C and EGTA to check possible non-specific inhibition of G-6-Pase unrelated to phospholipid hydrolysis. This was always less than 5%. G-6-Pase was assayed in 0.1 M cacodylate buffer, pH 6.5, using 80 mM G-6-P and incubating 20 min at 37°. Phosphorus was assayed by the method of Allen [3] and protein by the Lowry method [4]. Experiments on the reactivation of phospholipid depleted G-6-Pase were performed using

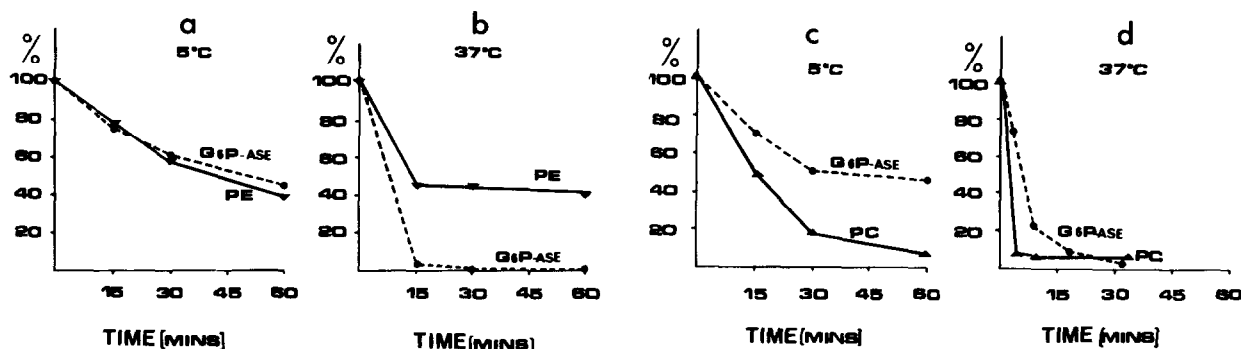


Fig. 1. Reaction mixtures were as in the text. Each reaction was stopped with EGTA and sampled for G-6-Pase activity and counts. In all cases a blank tube was run in which EGTA was added at zero time and the tube was then incubated like the experimental. Activity of G-6-Pase ( $\mu$ moles G-6-P hydrolysed/min/mg protein) and counts in these blank tubes were as followed: (a) 0.33, 3194 cpm ( $50 \mu\text{Ci } ^3\text{H-choline/160 g rat}$ ); (b) 0.35, 1345 cpm ( $25 \mu\text{Ci } ^3\text{H-ethanolamine/160 g rat}$ ); (c) 0.35, 3108 cpm ( $50 \mu\text{Ci } ^3\text{H-ethanolamine/160 g rat}$ ); (d) 0.34, 510 cpm ( $25 \mu\text{Ci } ^3\text{H-choline/300 g rat}$ ).

sonicated suspensions of lecithin [2]. This was prepared by sonicating lecithin at a concentration of approximately 7 mg per ml in 0.2 M tris-maleate buffer, pH 6.8, with an M.S.E. Ultrasonic Disintegrator at maximum power for 5–10 min. Samples were cooled in ice and kept under nitrogen throughout. The egg yolk and ox brain lecithins were shown to be at least 99% pure by thin layer chromatography before and after sonication. This phospholipid concentration of the final suspension was determined by total phosphate estimation. Radioactive choline labelled lecithin and ethanolamine labelled PE were counted in acid washed microsomal precipitates on glass fibre discs as described previously [5]. In both cases at least 97% of the incorporated radioactivity was in PC [5] or PE [6] respectively.

### 3. Results

In the presence of EGTA which removes the calcium essential for the action of *Cl. welchii* phospholipase C there was no significant hydrolysis of phospholipid nor loss of G-6-Pase activity. However, with calcium present at  $37^\circ$ , 90–100% of the PC (fig. 1d) and 50–70% of the PE (fig. 1b) were hydrolysed in 30 min. At this temperature the decrease in G-6-Pase activity most resembled the disappearance of lecithin. However, on  $5^\circ$  treatment the position differed. At this temperature, although the rate of hydrolysis was slower, the final degree of hydrolysis of PE and PC (figs. 1a and 1c) was the same as at  $37^\circ$ . However, the G-6-Pase activity

(figs. 1a and 1c) was totally different, not falling off to completion as previously shown, but instead plateauing at a level 40–60% of the original. Thus the decrease in the G-6-Pase activity resembled the decline in the PE counts and not that in the PC counts. If, however, an enzyme sample treated in the above manner (i.e. phospholipase at  $5^\circ$  followed by EGTA addition) was then exposed to a post-incubation at either  $20^\circ$  or  $37^\circ$ , the remaining G-6-Pase activity disappeared without any loss of counts. Thus the removal of phospholipid at low temperature not only renders the enzyme increasingly inactive but also progressively more heat labile. The difference in the G-6-Pase activity decline with  $37^\circ$  and  $5^\circ$  phospholipase treatment is therefore due to a proportional increase in heat instability with increased phospholipid depletion of the membrane. Accordingly, to study the direct phospholipid dependence of G-6-Pase by the use of phospholipase C, it is necessary to use low temperature incubation and thus remove the complicating heat lability factor. In view of this, it would appear that Duttera et al. [2] were studying the reactivation of heat inactivated G-6-Pase. Those authors using  $20^\circ$  phospholipase treatment found that added PE and lysolecithin reactivated G-6-Pase, while PC did not. We have repeated this work using two pure samples of PC from different sources (ox brain and egg yolk). With both these lecithins complete reactivation could be demonstrated if they were added after  $5^\circ$  phospholipase C treatment and before post-incubation at  $37^\circ$ . In addition, both conferred stability when the enzyme

Table 1

Except where stated, all systems had previously been exposed to phospholipase C treatment at 5° for 1 hr. The reaction was stopped by the addition of EGTA. In all the tubes where lecithin was not added it was replaced by an equivalent volume of tris-maleate buffer. Samples for G-6-Pase assays and counts were taken before and after postincubation. In all cases the counts did not decline during postincubation. The specific activity of G-6-Pase is expressed in  $\mu\text{moles P}_i/\text{min}/\text{mg}$  protein. The final PC concentrations were: ox brain lecithin 4.9 mg/ml; egg yolk lecithin 4.8 mg/ml. Similar post-incubation experiments at 20° for 0, 10, 20, 30 and 60 min gave complete inhibition of G-6-Pase by 30 min.

Microsomes	Specific activity after postincubation at 37°	
	0 min	10 min
Untreated	0.45	0.40
Treated	0.18	0.01
Treated*, plus ox brain lecithin	0.41	0.41
Treated*, plus egg yolk lecithin	0.39	0.39

\* If either sample of PC was added *after* 10 min postincubation at 37° no reactivation was obtained.

so treated was postincubated at 20° or 37° after PC addition. However, they were unable to reactivate heat inactivated G-6-Pase (table 1).

#### 4. Discussion

It appears that PC can reactivate G-6-Pase which has been simply depleted of phospholipid, while lysolecithin or PE [2] are required to reactivate enzyme which has become heat inactivated consequent upon phospholipid depletion. However, the persistence of

40–60% of the control G-6-Pase activity, when little PC remains in microsomes treated at 5° with phospholipase C, suggests that PC alone, probably does not play an essential role in G-6-Pase activity though it appears very important for stabilizing the active enzyme. It may be that the ability of PC to reactivate phospholipid depleted G-6-Pase is due to the potentiation of other phospholipids, possibly PE.

The close correlation in our studies between the decline in G-6-Pase activity and in PE at 5°, coupled with the apparent ability of PE to reactivate heat-inactivated G-6-Pase [2] support, but do not prove, an important role for PE. However, this putative role cannot require more than the 30–40% of PE remaining after phospholipase C treatment. Experiments are continuing to quantitatively define the role of PE with regard to G-6-Pase activity.

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